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Use of adipose tissue-derived stromal cells for prevention of esophageal stricture after circumferential EMR in a canine model

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Background: EMR is an accepted treatment for early esophageal carcinoma. However, resection of a large mucosal area often causes postoperative esophageal stricture.

Objective: To investigate the efficacy of autologous adipose tissue–derived stromal cells (ADSCs) for prevention of stricture formation after EMR in dogs.

Design: Animal study.

Setting: University research center.

Intervention: Ten beagle dogs were randomized into a control group and an ADSCs-injected (ADSC) group. The ADSCs were isolated from autologous adipose tissue. Immediately after circumferential esophageal EMR, about 5×10^6 ADSCs suspended in 8 mL of phosphate-buffered saline solution were injected endoscopically into the residual submucosa of the ADSC group, whereas the control group received only 8 mL of phosphate-buffered saline solution.

Main Outcome Measurements: Dysphagia score, weight loss, rate of mucosal constriction, and histologic assessments.

Results: In the control and ADSC groups, the median dysphagia scores were 4 and 1 ($P < .043$), the mean degrees of mucosal constriction were 75.7% and 45.3% ($P < .008$), and the numbers of nascent microvessels in the submucosal layer were 7.4 and 16.2 per unit area ($P = .007$), respectively. Atrophy and fibrosis of the muscularis propria layer were observed in the control group.

Limitations: Animal study, small sample size.

Conclusion: Injection therapy with autologous ADSCs suppresses constriction of the esophageal mucosa and improves clinical symptoms after circumferential EMR in this canine model. (Gastrointest Endosc 2011;73:777-84.)

Abbreviations: ADSCs, adipose tissue–derived stromal cells; BM-MSCs, bone marrow–derived mesenchymal stromal cells; CM-DiI, chloromethyl benzamido; HPF, high-power field; PBS, phosphate-buffered saline.

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Recent developments in therapeutic techniques for early esophageal cancer, based on the use of endoscopy, have gained widespread acceptance and brought benefits to a number of patients as a minimally invasive alternative to open surgery.^{1,2} In particular, endoscopic submucosal dissection makes it possible to achieve en bloc resection regardless of tumor size, even in cases of circumferential lesions.³ However, endoscopic therapy for superficial spreading lesions removes a large area of the esophageal mucosa, and as a result, patients often develop severe dysphagia^{4,5} and need to undergo periodic dilatation or placement of a temporary stent.⁶⁻⁸ Such mucosal defects can lead to deep ulceration, inflammation, and fibrosis of the esophageal wall. This reduces the elasticity and compliance of the esophagus, creating a greater tendency for the development of postoperative cicatricial stricture. As yet, no effective preventive measures or treatment for this serious complication have been

established. Compared with advances in technology for mucosal resection, currently available therapeutic measures remain inadequate for the treatment of mucosal defects after endoscopic therapy.

On the other hand, recent advances in autologous stromal cell therapy have facilitated its clinical application for organ and tissue regeneration. Studies using bone marrow–derived mesenchymal stromal cells (BM-MSCs) and adipose tissue–derived stromal cells (ADSCs) have been successful in regenerating various tissues such as cardiac muscle,^{9–11} blood vessels,¹¹ skin,^{12–14} and nerves.¹⁵ Some reports also have indicated that these cells may help to ameliorate various disease conditions by suppression of chronic tissue inflammation and abnormal fibrosis, like those observed in hepatic cirrhosis,^{16–18} pulmonary fibrosis,¹⁹ and vocal cord scarring.²⁰ This capacity seems to be attributable to the multilineage differentiation potential of these cells and their marked ability to secrete cell growth factors that can promote the repair of injured tissue and improve the quality of tissues that are regenerated.^{11,16–20}

We have hypothesized that such beneficial effects of ADSCs could be exploited to promote the process of wound healing in the esophageal wall after resection of a large area of mucosa, thus helping to prevent postoperative esophageal stricture. In the present study, we examined the preventive effect of ADSCs on esophageal stricture clinically and histologically after injection of autologous ADSCs into mucosal defects after circumferential EMR in a canine model by using devices equivalent to those used clinically in human patients.

MATERIAL AND METHODS

Animals and anesthesia

Ten beagle dogs (age <2 years; mean \pm SD) body weight 9.5 ± 1.6 kg) were randomly assigned to a control group (n = 5) or an ADSCs-injected (ADSC) group (n = 5). They were premedicated by intramuscular administration of atropine sulfate at 0.05 mg/kg. They were then anesthetized with 15 mg/kg ketamine hydrochloride and 3 mg/kg xylazine hydrochloride and intubated endotracheally. Halothane and nitrous oxide gas were used for maintenance of anesthesia during the procedure, under mechanical ventilation.

Isolation and labeling of ADSCs

In the ADSC group, autologous ADSCs were harvested from the anesthetized dogs 1 day before EMR. Approximately 30 mL of adipose tissue was obtained from the abdominal subcutaneous fat or the omentum. The adipose tissue was minced into small pieces (approximately 1 cm³) and digested in 20 mL of buffer containing 3 mg/mL type VIII collagenase (40% ammonium sulfate fraction from *Clostridium histolyticum*; Sigma Chemical Co, St. Louis, Mo) with intermittent shaking in a water bath at 37°C for 60 minutes. Collagenase was inactivated with an equal volume of

Take-home Message

- Advances in the prevention and therapy of esophageal stricture after large mucosal resection have extended the indication for endoscopic treatment for patients with esophageal carcinoma who are in poor physical condition.

Dulbecco modified Eagle medium containing 10% fetal bovine serum. After filtration through a 100- μ m filter and centrifugation (300g, 5 minutes), the floating fat and adipocytes were removed. The resulting pellets were resuspended in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco BRI, Grand Island, NY). Cells were labeled with chloromethyl benzamido (Cell Tracker CM-DiI; Invitrogen, Carlsbad, Calif) at a concentration of 1 μ g of CM-DiI per 1 mL of phosphate-buffered saline (PBS) solution (Sigma). CM-DiI–labeled cells were detected by using a fluorescence microscope (BZ-9000, Keyence, Japan). After the cells were incubated overnight at 37°C in a humidified atmosphere containing 5% carbon dioxide, they were harvested with 0.25% trypsin/1 mM ethylenediaminetetraacetic acid solution. For autologous injection, the cells were suspended at a concentration of 5×10^6 cells in 8 mL of PBS solution.

EMR procedure and intralesional injection of ADSCs

With each dog under general anesthesia, circumferential EMR was performed in the esophagus between 25 cm and 30 cm from the dental arch (Fig. 1A) by using an Olympus endoscopic system (GIF-XQ240; Olympus Optical Co, Ltd, Tokyo, Japan). Piecemeal mucosal resections were performed sequentially until a 5-cm circumferential resection had been achieved. All EMR procedures were performed by using normal saline solution as a submucosal injection solution, a cap with an outer diameter of 18 mm, a high-frequency-wave snare, and a generator with an automatically controlled system (ENDOCUT mode 120 W, Erbotom ICC 200; ERBE Elektromedizin GmbH, Tübingen, Germany). Immediately after EMR, about 5×10^6 ADSCs in 8 mL of PBS solution were injected into the residual submucosa in the ADSC group, whereas the controls each received only 8 mL of PBS solution. Injection of ADSCs into the submucosa was carried out endoscopically by using 25-gauge endoscopic sclerotherapy needles (Olympus). We injected 1 mL of the ADSCs suspended in PBS solution into each of 8 spots, the total amount being 8 mL, at equal intervals into 4 quadrants at both the oral and the anal sides in the exposed submucosal layer of the esophageal wall after EMR (Fig. 1B).

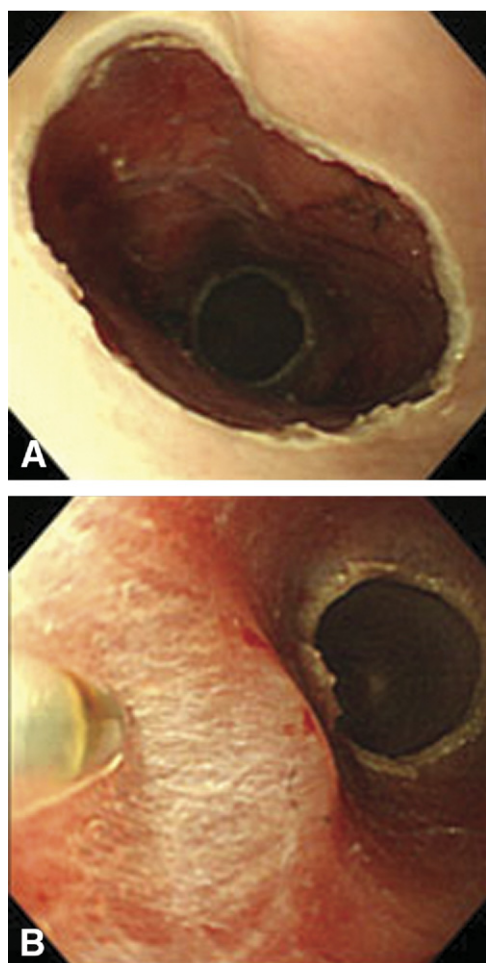


Figure 1. Circumferential EMR and adipose tissue–derived stromal cells (ADSCs) injection. **A**, A 5-cm circumferential mucosal defect was prepared in the thoracic esophagus. **B**, ADSCs were injected into the remaining submucosal layer.

Postoperative care

The dogs were fasted on the day of EMR and on the day after, and then liquid food was given after postoperative day 2. After postoperative day 5, the animals were fed a solid diet. If the dogs showed difficulty in taking the solid diet during the observation period, they were fed a semi-solid or liquid diet, depending on their conditions. If the animals were unable to take liquid, nutritional support at 60 kcal/kg/d was infused through a central venous line or gastric tube. Endoscopic examination was performed every 2 weeks. After the animals had been killed at postoperative week 8, macroscopic and histological evaluations were conducted. All experiments were done in accordance with protocols approved by the Ethics Committee for Animal Research of Kyoto University, Japan. Animal care, housing, and surgery were conducted in accordance with the rules and regulations of the committee.

Evaluation of esophageal stricture

Clinical evaluation. The rates of weight loss were calculated from body weight measured before EMR and immediately before killing and were compared between the control and ADSC groups. Dysphagia was scored by using a standard 5-point scale²¹ in a completely blinded manner: 0 = normal swallowing (able to swallow a solid diet), 1 = unable to swallow a proportion of the solid diet, 2 = able to swallow a semi-solid diet, 3 = able to swallow liquids only, and 4 = complete dysphagia including saliva.

Macroscopic evaluation. After an animal was killed, the esophagus was removed, dissected longitudinally, and examined macroscopically. The degree of stricture at the lesion site was expressed as the lateral mucosal contraction rate calculated by using the following equation, and the rates were compared between groups.

Mucosal contraction rate (%) =

$$\left\{ 1 - \frac{\text{Length of short axis at site of maximal contraction}}{(\text{Length of short axis at a normal mucosal site on upper side} + \text{Length of short axis at a normal mucosal site on lower side}) \times \frac{1}{2}} \right\} \times 100$$

Histological evaluation. The esophageal specimens were fixed in 10% formalin for 48 hours and were cut longitudinally to prepare paraffin blocks. Each section was examined after hematoxylin-eosin and Masson trichrome staining. In the ADSC group, the dynamics of injected cells labeled with CM-DiI were examined by fluorescence microscopy. Evaluation of angiogenesis and damage to the muscularis propria in all specimens was performed by a single investigator who was completely blinded to the study protocol.

Angiogenesis. The numbers of nascent microvessels in the lesion submucosal layer were compared between the control and ADSC groups. Five visual fields ($\times 200$ magnification) were selected in each specimen, and the number of nascent microvessels in each was counted. The average numbers of microvessels in each group were then calculated and compared.

Damage to the muscularis propria. Damage to the muscularis propria was assessed by using the following 4-step scoring system: 0 = no atrophic or fibrotic change in the muscularis propria evident in any of the examined sections, 1 = atrophy or fibrosis present but confined to the inner circular muscle layer, 2 = atrophy or fibrosis present but confined to the outer longitudinal muscle layer, and 3 = transmural fibrosis of the muscularis propria.

Statistics

The sample size of this study was determined based on the animal experiment guideline of Kyoto University, which is minimizing the number of animals used while

Table 1. Study outcomes

Group	Dog	Sex	Weight loss (kg) (ratio of body weight [%])	Dysphagia score	Mucosal constriction (%)	Muscularis propria damage score	Survival time (wk)	Cause of death
Control								
	1	M	1.8 (18.9)	4	71.7	3	8	Killed
	2	M	2.2 (26.2)	3	78.8	3	8	Killed
	3	F	1.9 (18.1)	1	53.8	2	8	Killed
	4	F	2.4 (27.0)	4	89.7	3	6.6	Aspiration pneumonia
	5	M	2.9 (32.2)	4	84.6	3	4.3	Aspiration pneumonia
ADSC								
	6	M	1.2 (13.3)	2	51.9	1	8	Killed
	7	F	0.5 (4.5)	0	42.9	0	8	Killed
	8	M	-0.6 (-4.8)	0	34.5	1	8	Killed
	9	M	2.4 (23.5)	3	65.2	1	8	Killed
	10	M	1.7 (15.4)	1	41.8	0	8	Killed

M, Male; F, female; ADSC, adipose tissue–derived stromal cells–injected.

allowing attainment of the scientific objective. With the risk of type I error and statistical power set at 0.05% and 80%, respectively, sample size was estimated with consideration of the efficacy of this treatment. All data were expressed as median (interquartile range) or mean \pm standard deviation (SD). Normally distributed variables were compared by using a *t* test, and non-normally distributed variables were compared by use of the Mann-Whitney *U* test. Differences at $P < .05$ were considered significant.

RESULTS

Circumferential EMR was performed safely in all dogs. The study outcomes are shown in Table 1. Because dogs 6 and 8 had only a small amount of subcutaneous fat, they were subjected to a 4-cm incision in the abdomen and resection of the omentum.

Clinical evaluation

Although one dog in the ADSC group developed a relatively severe stricture, it was able to take liquids. In the control and ADSC groups, the mean (\pm SD) degrees of weight loss were $24.5\% \pm 5.9\%$ and $10.5\% \pm 10.8\%$ ($P = .034$), and the median of dysphagia scores was 4 (1) and 1 (2) ($P = .043$), respectively. Figure 2 shows the endoscopic findings in the two groups. At postoperative day 14, severe ulceration and stricture were observed in the control group, whereas the lumen of the esophagus was maintained in the ADSC group (Fig. 2A and C). At 2 months after EMR, pinhole-sized stricture was evident in the control group. Although scar formation was evident,

and the lumen had narrowed slightly, the stricture in the ADSC group was mild (Fig. 2B and D).

Macroscopic appearance

Figures 3A and B show esophageal specimens excised from animals in the control and ADSC groups. The mean (\pm SD) rate of mucosal contraction was $75.7\% \pm 14.0\%$ in the control group and $47.2\% \pm 11.8\%$ in the ADSC group ($P = .008$), contractions being significantly milder in the ADSC group.

Histological findings

The histological appearances in the central parts of the lesions are shown in Figures 4A and B. In the control group, extensive destruction of the muscularis propria was evident. All layers of the muscularis propria had been penetrated and replaced by fibrous tissue. On the other hand, atrophic change and fibrosis in the muscularis propria were mild in the ADSC group. The median of damage scores for the muscularis propria was 3 (0) in the control group and 1 (1) in the ADSC group ($P = .009$). Many injected CM-DiI–labeled cells were found in the submucosal layer (Fig. 4C), although their differentiation to non-mesenchymal lineages was not clearly evident. Figures 4D and E show high-power fields (HPFs) in the submucosal layer. The mean (\pm SD) number of nascent microvessels was 7.4 ± 3.4 per HPF in the control group and 16.2 ± 4.4 per HPF in the ADSC group, being significantly higher in the latter ($P = .007$) (Fig. 4F).

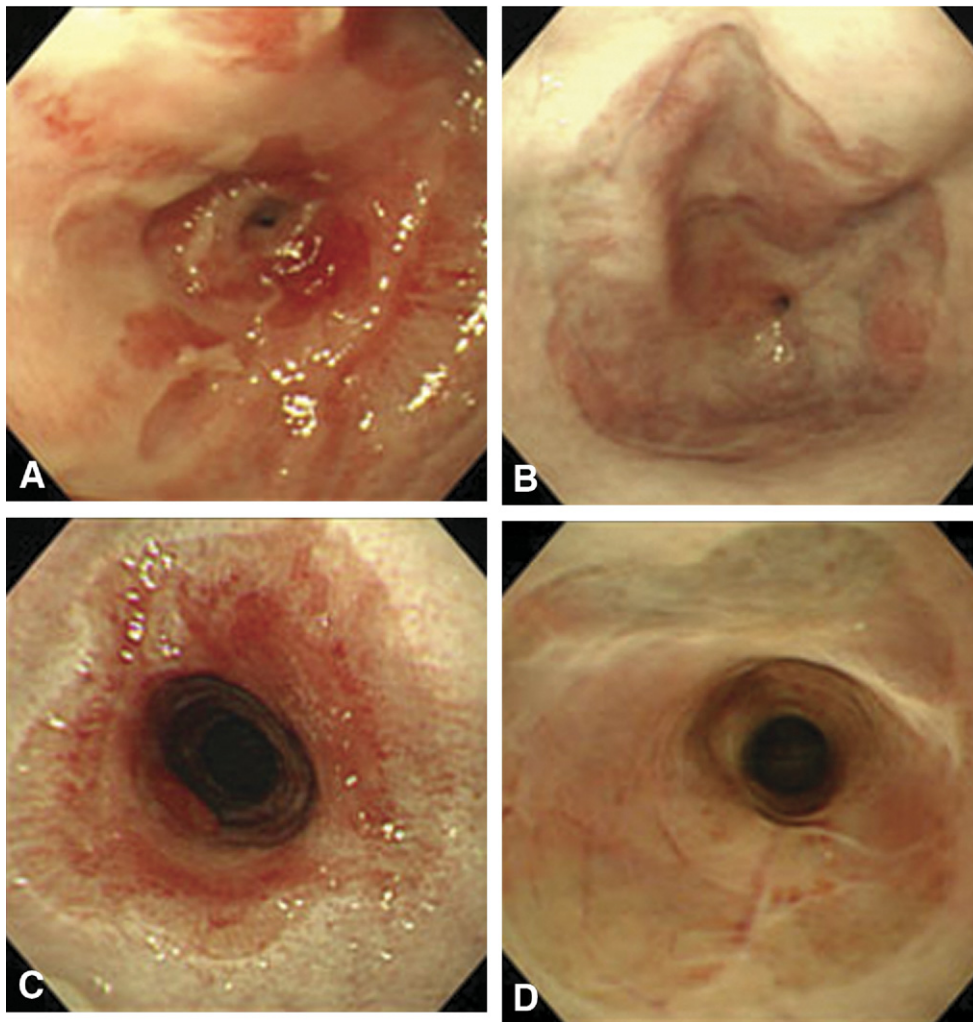


Figure 2. Comparison of the healing process. **A**, Dog 1 in the control group: Severe ulceration and stricture were observed at postoperative day 14. **B**, Dog 1: A pinhole-sized stricture was formed in the second month after EMR. **C**, Dog 7 in the adipose tissue–derived stromal cells–injected group: The lumen of the esophagus was maintained at postoperative day 14. **D**, Dog 7: The esophageal stricture was clearly improved, although scar formation and slight luminal narrowing were evident.

DISCUSSION

In this study, dogs injected with ADSCs had a significantly low mucosal contraction rate, less severe injury to the muscularis propria, and clinically mild dysphagia in comparison with those in the control group. These results indicated that injection of autologous ADSCs into the mucosal deficits after circumferential EMR was effective for prevention of postoperative stricture and improvement of clinical symptoms.

Therapeutic approaches for benign esophageal stricture have included dilatation with wire-guided bougies or balloons, temporary stent placement, and local injection of steroids. As shown in Figure 4A, esophageal stricture is caused by destruction and fibrosis of the muscularis propria. A fibrillized esophageal wall is vulnerable to damage and carries an increased risk of

perforation during treatment.^{6,7} Therefore, attention should be focused on stricture prevention after esophageal EMR. To prevent esophageal stricture, inflammation in mucosal defects should be minimized to suppress damage to the muscularis propria and prevent excess fibrosis. Prevention of esophageal stricture requires not only simple dilatation but also regenerative treatment to construct tissue that closely resembles that of the native esophagus.

Recently, the use of ADSCs has proved beneficial for treatment of various diseases and has been reported to improve the quality of regenerated tissues. For example, ADSC therapy for full-thickness skin defects,¹² radiation damage,²² vasculogenic ulcers,¹¹ and vocal cord scarring²⁰ has been shown to ameliorate abnormal fibrosis and to assist regeneration of tissues that closely resemble the native structure. It also has been reported that

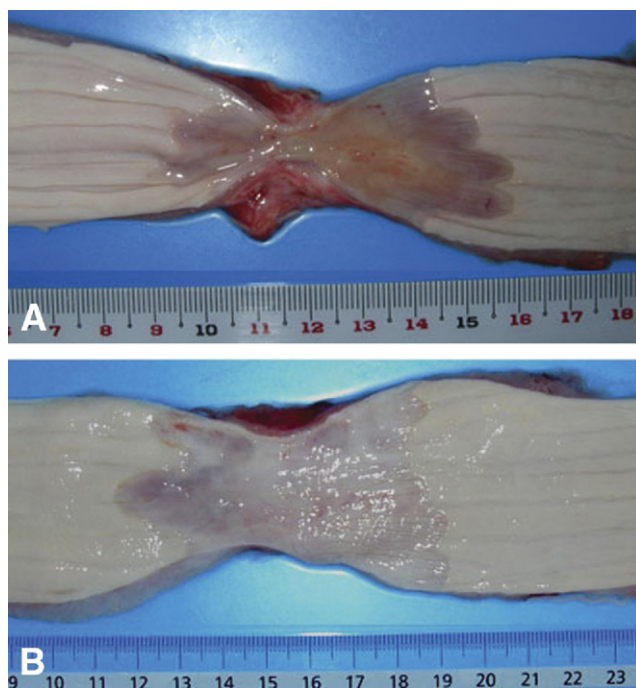


Figure 3. Macroscopic appearance. **A**, Dog 5 in the control group: the specimen showed severe mucosal contraction. **B**, Dog 8 in the adipose tissue-derived stromal cells-injected group: the mucosal contraction of the lesion was ameliorated.

portal vein administration of ADSCs for liver cirrhosis ameliorates liver fibrosis.¹⁸ In addition, the clinical application of ADSC treatment has been extended to fistulas in Crohn disease²³ and also myocardial infarction.¹¹

ADSCs demonstrate stem cell-like extensive self-renewal and are able to undergo differentiation into both mesenchymal (adipogenesis, chondrogenesis, osteogenesis) and nonmesenchymal (endothelial, smooth muscle, neurogenic) lineages. Moreover, ADSCs in standard culture secrete high levels of hepatocyte growth factor, vascular endothelial growth factor, placental growth factor, transforming growth factor-beta, fibroblast growth factor, granulocyte-macrophage colony-stimulating factor, monocyte chemotactic protein-1, and stromal-derived factor-1a, suggesting an important role of ADSCs in neovascularization.^{11,24,25} These functions of ADSCs are considered to affect keratinocyte-mesenchymal cell interaction,^{26,27} to improve the quality of regenerated tissues, and to suppress excessive fibrosis. In fact, in the present study, the ADSC group showed construction of tissue that was rich in nascent blood vessels and suppression of destruction and fibrosis in the muscularis propria. It is suggested that injected autologous ADSCs exhibit beneficial effects such as those described herein and can ameliorate esophageal stricture. However, the biochemical and molecular biological mechanisms underlying these phenomena have not

been elucidated in detail. If ADSCs are injected after incomplete resection, they may increase the risk of tumor growth and metastasis, and this has recently been an issue of contention. Previous studies have demonstrated both tumor-promoting and tumor-suppressive effects of ADSCs or BM-MSCs on some human tumor cell lines, both in vitro and in vivo,²⁸ although no report has described any relationship between esophageal cancer and ADSCs. The effects of stromal cell therapy on tumor proliferation vary according to the tumor type. For example, ADSCs promote the proliferation of breast cancer, melanoma, and prostate cancer cells²⁹⁻³¹ but inhibit the growth of pancreatic adenocarcinoma, lung carcinoma, and Kaposi sarcoma cells.³²⁻³⁴ As a first step toward adopting this method for human clinical use, evaluation in benign diseases such as corrosive or peptic esophagitis might be suitable, although in the meantime, the roles and influences of ADSCs on esophageal cancer cells will need to be investigated in detail.

Some studies have reported the application of regenerative skin treatment to the esophagus. Nieponice et al³⁵ demonstrated that insertion of a xenogenic acellular matrix from the urinary bladder exerted a preventive effect against esophageal stricture due to mucosal defects in dogs. Other animal studies have reported a method using a cell sheet prepared from autologous cultured oral mucosal epithelial cells.³⁶ Combination of such a scaffold, an epithelial cell sheet, and injection of autologous ADSCs might further improve the clinical outcome. Many reports have documented tissue regeneration by using BM-MSCs, which are stromal cells with properties similar to those of ADSCs. However, unlike BM-MSCs, ADSCs can be obtained easily and safely in large numbers from subcutaneous adipose tissue in several regions of the body by using liposuction, without the need to culture and expand them to obtain a therapeutic dose. A typical harvest of 100 mL of human adipose tissue contains about 1 to 5×10^6 nonbuoyant stromal cells, about 40-fold more than a typical harvest of BM-MSCs (normally 2.5×10^4 cells in a 40-mL volume) from a mature adult.²⁶

In the present study, ADSCs were isolated 1 day before the EMR procedures to confirm CM-DiI labeling. In a clinical setting, ADSCs can be harvested and isolated simultaneously with endoscopy treatment by using the Celution system (Cytori Therapeutics Inc., San Diego, CA).³⁷ With this method, isolated ADSCs can be injected immediately after EMR, and cell alterations during cell culture can be avoided.

Advances in the prevention and therapy of esophageal stricture after extensive mucosal resection have extended the possibilities of endoscopic treatment for patients in poor physical condition. Our present results show that our new technique is simple and safe and can improve clinical outcomes; furthermore, the information we have obtained will

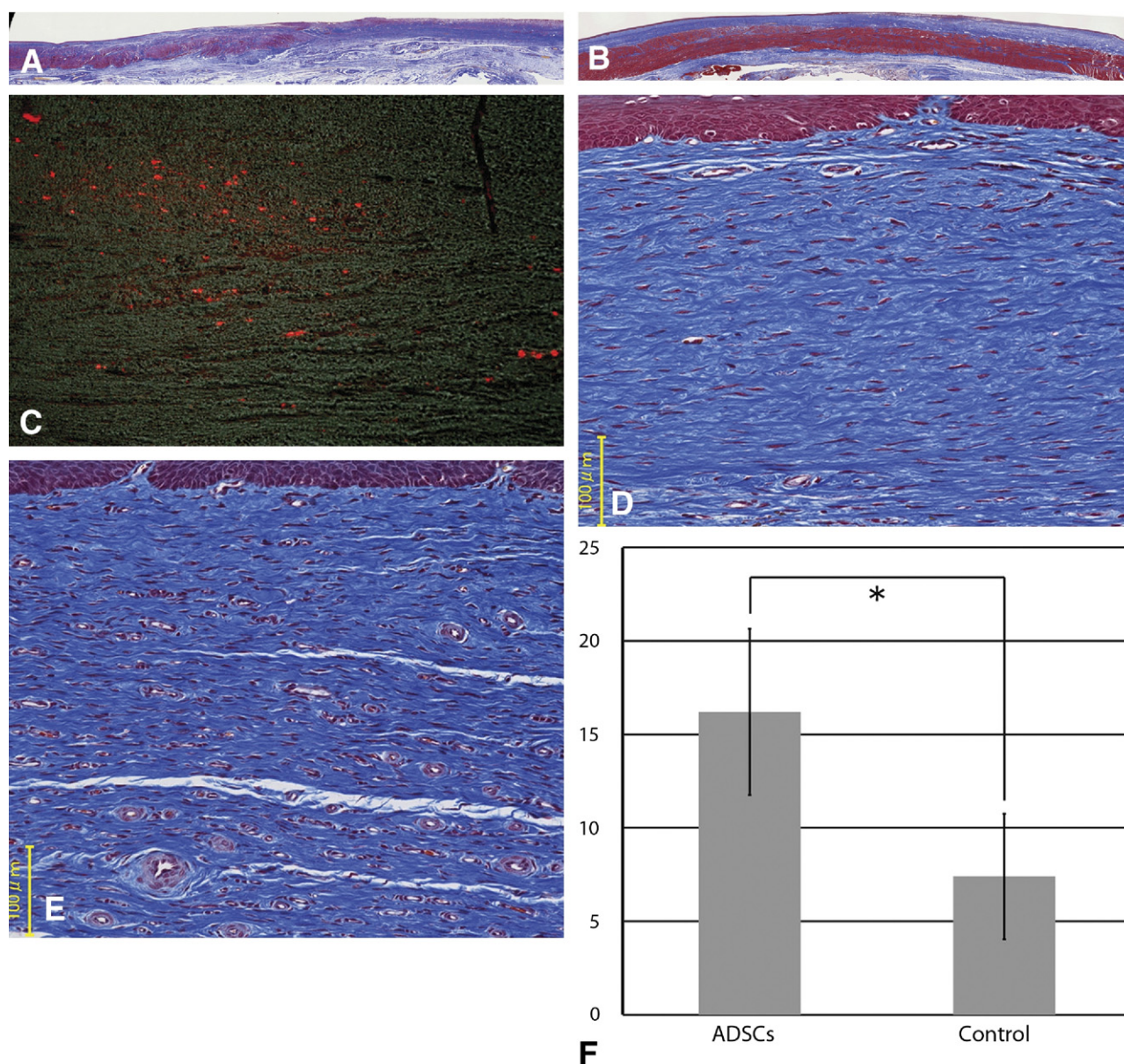


Figure 4. Histological findings. **A**, Dog 1: Transmural destruction of the muscularis propria is observed. Transmural damage of the muscularis propria is evident, and muscularis propria has been replaced by fibrosis (Masson trichrome, orig. mag. $\times 40$). **B**, Dog 9: There is little atrophy in the muscularis propria, although the dysphagia score was 3 (Masson trichrome, orig. mag. $\times 40$). **C**, Many injected chloromethyl benzamido-labeled cells showing a red color are evident in the submucosal layer. **D**, High-power fields of the submucosal layer in the control group. There are few microvessels among dense collagen fibers (Masson trichrome, orig. mag. $\times 200$). **E**, High-power fields in the ADSC group. Many microvessels are evident (Masson trichrome, orig. mag. $\times 200$). **F**, The number of nascent microvessels is 7.4 per 1 field ($\times 200$) in the control group and 16.2 in the ADSC group, the difference being significant (* $P = .007$). ADSC, adipose tissue-derived stromal cells-injected group.

be valuable for preclinical studies aimed at the prevention of esophageal stricture by injection of autologous ADSCs.

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